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Somatic transformation efficiencies and expression patterns using the *Jc*DNV and *piggyBac* transposon gene vectors in insects

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Abstract

A somatic transformation gene vector that exploits the genomic integration properties of *Junonia coenia* lepidopteran densovirus (*Jc*DNV) sequences *in vivo* has been developed. *Jc*DNV somatic transformation vectors are derivatives of plasmids containing an interrupted genome of *Jc*DNV that provide efficient, robust vectors that can be used to examine regulation of chromosomally integrated transgenes in insects. Microinjection of *Jc*DNV plasmids into syncytial embryos of *Drosophila melanogaster* or the lepidopterans *Plodia interpunctella*, *Ephestia kuehniella* or *Trichoplusia ni* resulted in persistent transgene expression throughout development. Inclusion of transgenes with tissue-specific promoters resulted in expression patterns canonical with phenotypes of *piggyBac* germline transformants. Somatic transformation required the presence of the viral inverted terminal repeat in *cis* only and did not depend upon non-structural viral proteins.

Keywords: genetic transformation, densovirus integration, transposable elements, *Drosophila*, *Plodia interpunctella*.

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Introduction

The advent of transposon-directed germline transformation in insects has provided the necessary biotechnology to begin to assess the utility of genetically modified insects as biocontrol agents carrying sterility or lethality genes (Alphey, 2002; Robinson *et al*., 2004; Marec *et al*., 2005) or to provide new avenues for biopharmaceuticals (Tomita *et al*., 2003). Although this biotechnology can already be used in species from four different orders (Robinson *et al*., 2004), it is not applicable to all species where transformation has been tested (Shirk, Perera & Bossin, unpublished data). The development of transgenesis in new species depends upon (1) the availability of reporter cassettes that are functional in the species being tested and (2) the utilization of a transposon vector that is mobile in this species. The development of transgenesis in a new species without addressing these requirements may lead to difficulties in progressing to the desired result. Additionally, even within those species where germline transformation is available, husbandry and screening of transgenics in large numbers is often prohibitive, particularly when multiple constructs must be assessed to establish the efficacy of the transgenes. Utilization of somatic transformation vectors that result in efficient chromosomal integrations should provide the biotechnology to rapidly test expression cassettes in G0 insects. This would permit promoter activity assessment and phenotype description without complications from achieving efficient germline transformation or conducting the requisite husbandry.

Stable somatic transformation of *Drosophila melanogaster* has been achieved following microinjection of syncytial embryos with plasmids containing the interrupted *Junonia coenia* lepidopteran densovirus (*Jc*DNV) genome (Royer *et al*., 2001). The *lac*Z coding sequence was inserted in frame within the capsid protein gene (VP) and expression of these chimeric proteins was detected throughout postembryonic development into the adult stage. Integration of *Jc*DNV sequences within genomic DNA from transformed adults was demonstrated by Southern blot analysis. Subsequent studies analysed *Spodoptera Frugiperda* SF9 cell lines stably transformed with a *Jc*DNV vector expressing green

fluorescent protein (GFP) and showed that the majority of the *Jc*DNV vector becomes integrated into the chromosomes (Bossin *et al*., 2003). Both Southern blot analysis and primed *in situ* synthesis showed vector DNA to be incorporated into SF9 genomic DNA and successful recovery of the vector was achieved by plasmid rescue following digestion of genomic DNA (Bossin *et al*., 2003). Additionally, isolation and sequencing of integration sites from SF9 genomic DNA demonstrated that the majority of recovered integrations involved the P9-associated inverted terminal repeat (P9ITR) (Bossin *et al*., 2003). The P9ITR includes the 517 bp ITR partially overlapping with the 73 bp P9 promoter, which regulates production of a 2.6 kb transcript coding for four capsid proteins (Dumas *et al*., 1992). However, none of the recovered integration sites involved the P93ITR (Bossin *et al*., 2003), which includes the P93 promoter that regulates production of 2.4 and 1.7 kb transcripts coding for three nonstructural proteins (Dumas *et al*., 1992).

The present study examines the utility of *Jc*DNV-derived plasmids as somatic transformation vectors in various insects of different orders and provides a comparison with the somatic transformation efficiency derived from the *piggyBac* transposon. Previous work relied upon identifying somatic transformants on the basis of a marker gene driven by the densoviral P9 promoter. Inclusion of additional expression cassettes within *Jc*DNV-derived vectors permitted assessment of expression patterns and provided a means of determining promoter activity and phenotype of transgenes in flies and moths. This work demonstrates that *Jc*DNV-derived vectors provide a convenient and efficient somatic transformation system for rapid and accurate assessment of gene activity in insects.

Results

Somatic transformation in Diptera and Lepidoptera

In order to determine whether *Jc*DNV somatic transformations result in canonical expression of transgenes incorporated in the vectors, the 3xP3EGFP expression cassette (Berghammer *et al*., 1999) was inserted into pJDsRed, a plasmid expressing the four chimeric VP-DsRed proteins (Fig. 1A). pJDR-3xP3G was microinjected into syncytial embryos of *D. melanogaster* and the expression pattern in larvae and adults was compared with pB[3xP3EGFP]af (Berghammer *et al*., 1999) germline-transformed *D. melanogaster* strains (Fig. 1B). While only 4% of pJDR-3xP3G microinjected eggs hatched as compared with 29% for buffer alone, the transformation rate of recovered larvae was 41%. In contrast, when embryos were microinjected with phr5IE1DsRed, a plasmid that does not contain sequences supporting recombination, only 0.5% of G0 larvae showed persistent DsRed expression (Table 1). Third instar larvae somatically transformed with pJDR-3xP3G were examined for GFP expression and compared with larvae from pB[3xP3EGFP]af germline-transformed *D. melanogaster* strains (Fig. 2B). GFP expression in pJDR-3xP3G somatic transformants was identical to that observed in pB[3xP3EGFP]af-transformed larvae. GFP could be observed in the Bolwig's organ, labial discs and nerves around the intestinal tract (Fig. 2A).

Canonical expression driven by the 3xP3 artificial binding site persisted through the adult stage where GFP was observed in the eyes of somatic transformants (Fig. 2D,E). GFP expression showed a variegated pattern, presumably as a result of the occurrence of multiple integrations that occurred within various cell lineages derived from embryonic tissues. DsRed expression could be observed in fat body throughout the adult body (Fig. 2C,D). Because the expression patterns arising from the two cassettes within the pJDR-3xP3G vector occur correctly in tissues of larvae, pupae (data not shown) and adults, these findings suggest that pJDR-3xP3G integrations must occur quickly following microinjection and involve the majority of nuclei.

The pJDR-3xP3G vector was microinjected into syncytial embryos of *Plodia interpunctella* (Lepidoptera: Pyralidae) to assess somatic transformation activity of the vector in a moth. DsRed expression was clearly observed in 39% of the embryos while only 7% showed detectable GFP (Table 2). No fluorescence was observed in buffer-microinjected embryos while 36% of phr5IE1DsRed-microinjected embryos showed DsRed fluorescence. The hatch rate of *P. interpunctella* embryos microinjected with pJDR-3xP3G was 27% as compared with 34% with buffer alone and 15% with the phr5IE1DsRed control plasmid. Of the larvae observed, 70% showed DsRed expression (Table 2). Only one of these larvae (0.02%) showed GFP expression concomitant with DsRed expression. None of the buffer- or phr5IE1DsRed-injected larvae showed any fluorescence. In pJDR-3xP3G transformants, DsRed expression was observed from the first instar through to the adult stage (Fig. 3). Somatically transformed second instar larvae showed DsRed expression throughout the body (Fig. 3C,E). The highest level of expression was in the cardiac cells that extend along the dorsal aorta (Fig. 3C). In live views of these transformed larvae, peristaltic pumping of the dorsal aorta was clearly observed (data not shown). In two larvae that also showed GFP expression, the pattern was apparent in the crop and along nerves surrounding the intestinal tract (Fig. 3D). DsRed expression persisted through the adult stage and could be observed in the eyes of adults (Fig. 3G,I). In a larva that had GFP expression, the adult also showed GFP expression in the eye (Fig. 3J).

Microinjection of pJDR-3xP3G into *Ephestia kuehniella* (Lepidoptera: Pyralidae) syncytial embryos resulted in expression patterns similar to those in *P. interpunctella*. Somatically transformed larvae had high levels of DsRed expression in cardiac cells and significant levels throughout the body (Fig. 4B,C).

The results were similar to the preceding observations when pJDR-3xP3G was microinjected into syncytial embryos

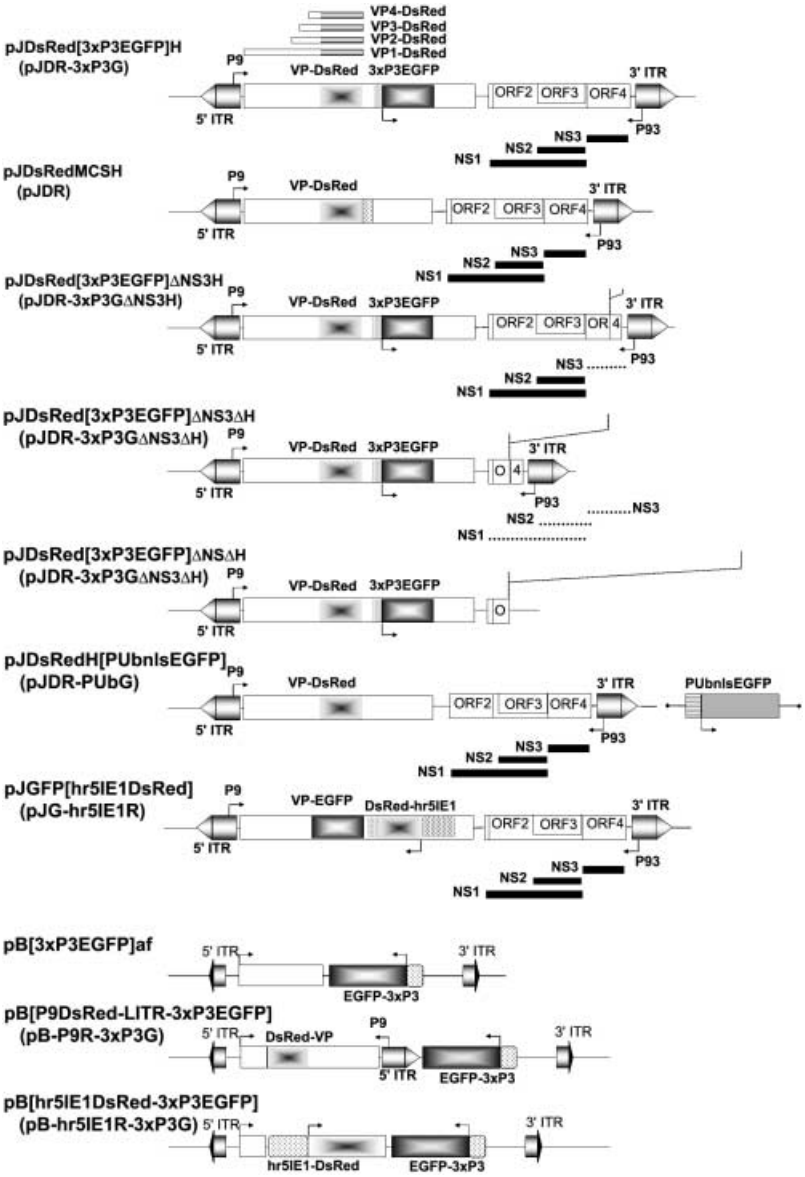

Figure 1. Organization of *Junonia coenia* lepidopteran densovirus (*Jc*DNV) (A) and *piggyBac* (B) transformation vectors. Each plasmid was constructed as described in the Experimental procedures. All *Jc*DNV plasmids were derived from pJGFPH (Bossin *et al*., 2003). The salient features of the *Jc*DNV genome and *piggyBac* transposon are designated within each diagram. Insertions of the 3xP3EGFP (Berghammer *et al*., 1999), Pub-nls-EGFP (Handler & Harrell, 2001), P9DsRed-LITR and hr5IE1EGFP expression cassettes are shown by shaded boxes. Open thick lines containing light grey shading represent the VP-GFP fusion proteins, solid thick black lines represent the NS proteins, dashed vertical Ys represent deletions and dotted lines show NS proteins that are no longer expressed. Bent arrows over promoters show the direction of transcription. Abbreviations: 5′-ITR, 5′ inverted terminal repeat; 3′-ITR, 3′ inverted terminal repeat; ORF, open reading frame; TT, *piggyBac* transposase.

of *Trichoplusia ni* (Lepidoptera: Noctuidae). Approximately 34% of the embryos showed DsRed expression while 47% had GFP expression (Table 2). DsRed expression was observed in 35% of embryos microinjected with the phr5IE1DsRed control plasmid but could be detected in only 0.5% of the larvae that hatched. Although the sample size was small, 24% of pJDR-3xP3G larvae showed DsRed expression but none showed concomitant GFP expression. As observed before, larvae somatically transformed with pJDR-3xP3G showed high levels of DsRed expression in cardiac cells as well as in the posterior fat body (Fig. 4D,E).

Effects of sequence modification on somatic transformation

The embryo somatic transformation assay was used as a basis to assess the importance of viral sequences and the influence of positional organization of expression cassette insertion sites within *Jc*DNV vectors on the efficiency of transformation. Insertion of a multiple cloning site adjacent to the SV40 polyadenylation sequence of the P9-DsRed expression cassette had no effect on the transformation rate in any insects tested. Microinjection of pJDR into *D. melanogaster* resulted in a 49% transformation rate (Table 1) compared with 80% in *P. interpunctella* (Table 2) and 31% in *T. ni* (Table 2). Substitution of EGFP for DsRed to produce pJDG did not affect the somatic transformation rate in *D. melanogaster* (76%).

It was previously reported that deletion of the NS3 gene (Royer *et al*., 2001; Bossin *et al*., 2003) or all of the non-structural genes (Bossin *et al*., 2003) had no apparent effect on achieving somatic transformation. However, there was no assessment of transformation efficiency in these studies. Similar deletions plus the deletion of the P93ITR

Table 1. Somatic transformation rates and effects of sequence modification of *Jc*DNV and *piggyBac* vectors in G0 *Drosophila melanogaster*

Vector*	No. eggs injected	Larvae observed	Somatic transformants†		Larval transient expression
			DsRed	GFP	
pJDR-3xP3G	6463	0 = 4.1%		0 = 41%	
(*n* = 8 trials)		(s = 5.6)		(s = 16)	
Buffer	337	97 (29%)		0 (0%)	
phr5IE1DsRed	1712	249 (15%)		1 (0.5%)	
pJDR	970	112 (12%)		55 (49%)	
pJDG	527	38 (7%)		29 (76%)	
pJDR-3xP3GΔNS3H	3289	138 (4%)		20 (14%)	
pJDR-3xP3GΔNSH	928	52 (6%)		4 (8%)	
pJDR-3xP3GΔNSΔH	798	32 (4%)		2 (4%)	
pJDR-PUbG	1333	57 (4%)		30 (52%)	
pJDG-hr5IE1R	1556	213 (14%)	27 (13%)	66 (31%)	
pB[3xP3EGFP]af‡	4089	ND	0		361 (9%)
pB-P9R-3xP3G‡	1448	ND	0		257 (18%)
pB-hr5IE1R-3xP3G‡	389	ND	0		59 (15%)

*Plasmid concentrations were 1 μg/μl for *Jc*DNV vectors and 600 : 400 ng/μl for *piggyBac* vectors and helper.
†Somatic transformation per cent determined on the basis of the larvae expressing GFP (G+) or DsRed (R+) per total G0 larvae observed.
‡Transient expression limited to first instar larvae.
ND, not determined.

were made utilizing pJDR-3xP3G as the starting construct (Fig. 1A). An *Nsi*I deletion resulting in elimination of the NS3 gene, pJDR-3xP3GΔNS3H, was microinjected into *D. melanogaster* and *P. interpunctella* syncytial embryos. Elimination of the NS3 gene did not significantly affect transformation efficiencies in *P. interpunctella* but did result in a decrease in efficiency in *D. melanogaster*. In *P. interpunctella* the transformation rate was 60% based on DsRed expression with only 2% showing concomitant GFP expression (Table 2), whereas in *D. melanogaster*, the somatic transformation rate was 14% and all transformants showed both DsRed and GFP expression (Table 1). Elimination of expression from all NS genes by partial deletion of NS coding sequences did not affect the transformation efficiency, nor did deletion of NS genes plus P93ITR in *P. interpunctella*. Transformation efficiencies were 78% for pJDR-3xP3GΔNSH (Table 2) and 40% for pJDR-3xP3GΔNSΔH. However, the transformation rate was 8% for pJDR-3xP3GΔNSH and 4% for pJDR-3xP3GΔNSΔH in *D. melanogaster* (Table 1). For these two deletions, none of the transformants in *P. interpunctella* showed 3xP3-driven GFP expression, while in *D. melanogaster*, GFP and DsRed were coexpressed in the transformants.

The effect of placing a secondary expression cassette within the viral sequences was assessed by inserting the hr5IE1DsRed cassette into the MCS of pJG. The hr5IE1 enhancer/promoter also provided a test of expression from the P9 promoter along with a second strong viral promoter. The dual-labelled vector was microinjected into syncytial embryos of both *D. melanogaster* and *P. interpunctella*. The larvae of *P. interpunctella* showed expression of both GFP (25%) from the P9 promoter and DsRed (32%) from the hr5IE1 promoter, demonstrating that both promoters were functional following somatic transformation with the *Jc*DNV vector. In G0 larvae of *D. melanogaster*, 31% showed P9-driven GFP expression and 13% showed hr5IE1-driven DsRed expression.

To test the impact of placing the secondary expression cassette outside the densovirus sequences as well as testing the efficiency of the *D. melanogaster* polyubiquitin promoter-EGFP expression cassette (PUb-nls-EGFP) (Handler & Harrell, 2001), pJDR-PUbG was microinjected into syncytial embryos of *D. melanogaster* and *P. interpunctella*. The transformation rate was not affected by placing the second expression cassette outside the densoviral sequences and the expression pattern presented a normal profile for polyubiquitin-regulated sequences (data not shown). In G0 larvae of *D. melanogaster*, 52% showed concomitant DsRed and GFP expression, while in *P. interpunctella* larvae, 37% showed DsRed expression alone. There was no apparent *D. melanogaster* polyubiquitin promoter-driven expression of GFP in *P. interpunctella* during postembryonic development.

Comparative somatic transformation efficiencies between Jc*DNV and* piggyBac

The *piggyBac* transposon has somatic (Fraser *et al.*, 1995) as well as germline (Handler *et al.*, 1998) transposition activity. To assess the utility of *piggyBac* as a somatic transformation vector, the P9DsRed and 3xP3EGFP expression cassettes used in *Jc*DNV vectors were introduced into the *piggyBac* vector pB[3xP3EGFP]af (Berghammer *et al.*, 1999) to produce pB-P9R-3xP3G and pB-hr5IE1R-3xP3G (Fig. 1B). Each vector with the phsp-pBacwc helper was comicroinjected into syncytial embryos of *D. melanogaster* and *P. interpunctella* and G0 larvae were scored for

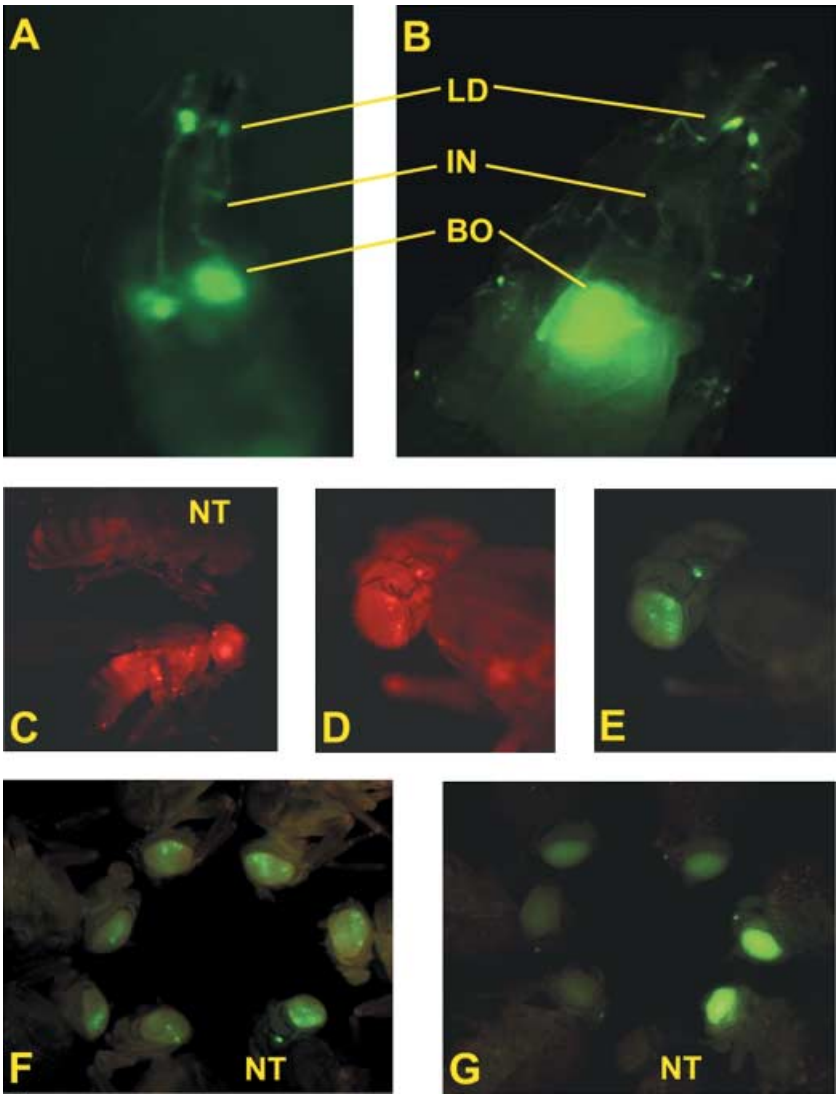

Figure 2. Comparison of the 3xP3-driven GFP expression pattern in somatic- and germline-transformed *Drosophila melanogaster*. G0 *D. melanogaster* larva somatically transformed with pJDR-3xP3G (panel A). G6 *D. melanogaster* larva that carries a pB[3xP3EGFP] insertion (panel B). GFP expression in Bolwig's organ, labial discs and the intestinal nerve net can be observed in both the somatic- and the germline-transformed strains. G0 *D. melanogaster* adults somatically transformed with pJDR-3xP3G showing whole body (panel C) and eye (panel D) expression of DsRed and eye (panel E) expression of GFP. The variegated pattern of GFP expression in seven pJDR-3xP3G somatically transformed pJDR-3xP3G adults (panel F). Variability in GFP expression levels in G6 *D. melanogaster* adults from six different transformed lines that carry pB[3xP3EGFP] insertions (panel G). Abbreviations: BO, Bolwig's organ; IN, intestinal nerves; LD, labial discs; NT, nontransformed control.

somatic transformation. Although expression from the constructs was detected in the first and second instar larvae of both species (Tables 1 and 2), expression was limited to these instars. Additionally, the expression pattern of the 3xP3 reporter cassette did not match the pattern observed with *piggyBac*-mediated germline transformations. Small localized patches of expression were observed in posterior tissues and GFP expression was observed in non-neural tissues (Fig. 3K). GFP observed in these larvae was probably transient expression derived from plasmids persisting in early larval instars and indicates that somatic integration of the *piggyBac* vectors was extremely rare when compared with that observed with *Jc*DNV vectors.

Discussion

Densoviruses were first discovered in the moth *Galleria mellonella* (Lepidoptera: Pyralidae) (Meynadier *et al*., 1964) and further described on the basis of their common properties with Parvoviridae as autonomously replicating insect parvoviruses (Tijssen & Bergoin, 1995). Members of the Densovirinae subfamily of Parvoviridae are pathogenic for insects, mainly Lepidoptera, Diptera and Orthoptera (Bergoin & Tijssen, 1998). Densoviruses share many characteristics with vertebrate parvoviruses such as adeno-associated viruses that are utilized as shuttle vectors for human gene therapy. Previous studies have examined the potential use of recombinant densoviruses as potential gene transfer systems in insects (Giraud *et al*., 1992; Corsini *et al*., 1996; Bergoin & Tijssen, 1998; Afanasiev & Carlson, 2000). These earlier approaches were based on the production of transducing recombinant viral particles that could vector foreign gene constructs. Some of these systems were successfully employed to vector genes. By inserting the *lac*Z coding sequence into the capsid gene of the *J. coenia* densovirus and then packaging the

Table 2. Somatic transformation rates and effects of sequence modification of *Jc*DNV and *piggyBac* vectors in G0 *Plodia interpunctella* and *Trichoplusia ni*

Vector*		No. eggs injected	No. fluorescent eggs		Larvae observed	Somatic transformants†		Larval transient expression	
			DsRed	GFP		DsRed	GFP	DsRed	GFP
pJDR-3xP3G	Pi	1663	654 (39%)	124 (7%)	441 (27%)	310 (70%)	1 (0.02%)		
	Tni	560	190 (34%)	265 (47%)	25 (4%)	6 (24%)	0 (0%)		
Buffer	Pi	295	0 (0%)		100 (34%)	0 (0%)	0 (0%)		
phr5IE1DsRed	Pi	4162	1514 (36%)		616 (15%)	0 (0%)			
	Tni	1712	595 (35%)		205 (12%)	1 (0.5%)			
pJDR	Pi	3454	1551 (45%)		553 (16%)	440 (80%)			
	Tni	2063	847 (41%)		215 (10%)	67 (31%)			
pJDR-3xP3GΔNS3H	Pi	2546	ND	ND	171 (7%)	103 (60%)	3 (2%)		
pJDR-3xP3GΔNSH	Pi	2595	ND	ND	153 (6%)	120 (78%)	0 (0%)		
pJDR-3xP3GΔNSΔH	Pi	1454	1358 (93%)	1109 (76%)	311 (21%)	124 (40%)	0 (0%)		
pJDR-PUbG	Pi	2342	1779 (75%)	0 (0%)	288 (12%)	106 (37%)	0 (0%)		
pJDG-hr5IE1R	Pi	1070	ND	ND	57 (5%)	18 (32%)	14 (25%)		
pB[3xP3EGFP]af‡	Pi	1570		1324 (84%)	333 (21%)		0 (0%)		151 (45%)
pB-P9R-3xP3G‡	Pi	493	253 (51%)	157 (32%)	64 (25%)	0 (0%)	0 (0%)	60 (94%)	0 (0%)
pB-hr5IE1R-3xP3G‡	Pi	839	711 (85%)	711 (85%)	252 (60%)	0 (0%)	0 (0%)	22 (9%)	47 (19%)

*Plasmid concentrations were 1 μg/μl for *Jc*DNV vectors and 600 : 400 ng/μl for *piggyBac* vectors and helper.
†Somatic transformation per cent determined on the basis of the larvae expressing GFP or DsRed per total G0 larvae observed.
‡Transient expression limited to first instar larvae.
Pi, *Plodia interpunctella*; Tni, *Trichoplusia ni*.
ND, not determined.

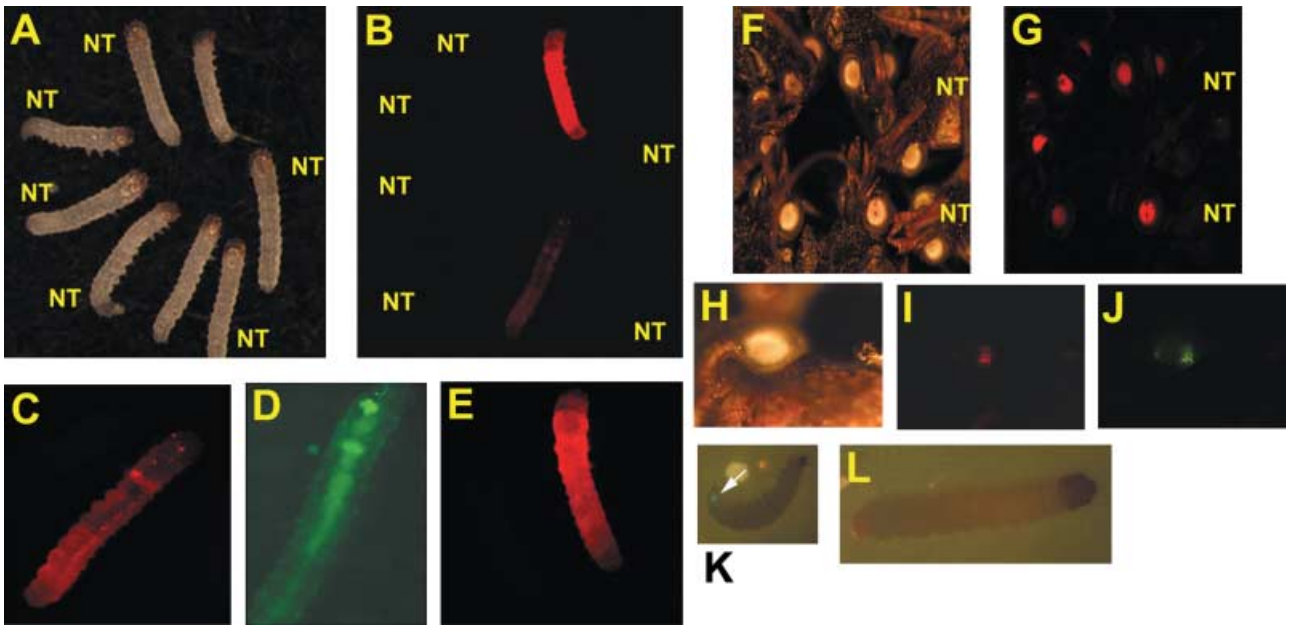

Figure 3. Pattern of P9-driven DsRed and 3xP3-driven GFP expression in *Plodia interpunctella* larvae somatically transformed with pJDR-3xP3G and compared with transient expression of GFP in G0 *P. interpunctella* larvae microinjected with pB[3xP3EGFP]af. Six normal and two somatically transformed second instar larvae of *P. interpunctella* viewed with bright field (panel A) and Texas red filter (panel B). The two somatically transformed larvae can be observed in the right-hand sector of the field. The background autofluorescence in normal larvae cannot be observed in this view because of the very high level of fluorescence in the somatically transformed larvae. Higher magnification views of the two somatically transformed larvae show DsRed expression in tissues from the anterior to the posterior (panels C and E). A high level of DsRed expression can be observed in the cardiac cells along the dorsal aorta (panel C). 3xP3-driven GFP expression (panel D) can also be observed in the larva in panel C. Two normal and six somatically transformed adults of *P. interpunctella* viewed with bright field (panel F) and Texas red filter (panel G). The two nontransformed adults can be observed in the right-hand sector of the field. Higher magnification views of a somatically transformed adult showing DsRed (panel I) and GFP (panel J) expression in the eye of a transformed adult (bright field, panel H). A pB[3xP3EGFP]af somatically transformed G0 *P. interpunctella* larva at first instar showing an inappropriate GFP expression (panel K, arrow) and a last instar (panel L) showing the temporal loss of GFP expression during larval development. Abbreviation: NT, nontransformed control.

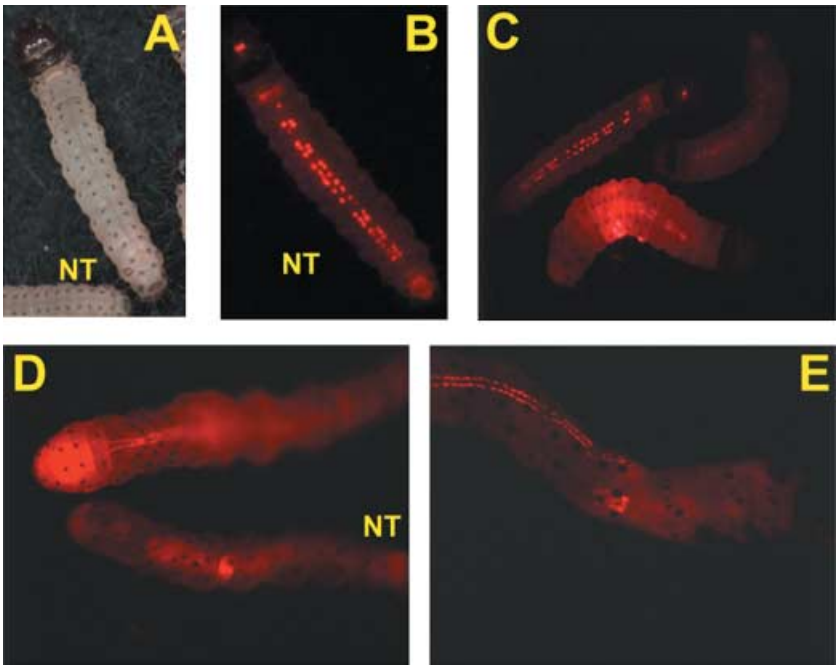

Figure 4. Pattern of P9-driven DsRed expression in *Ephestia kuehniella* and *Trichoplusia ni* larvae somatically transformed with pJDR. A somatically transformed second instar larvae of *E. kuehniella* viewed with bright field (panel A) and Texas red filter (panel B). The high level of DsRed expression in the cardiac cells along the dorsal aorta in transformed larvae can be easily observed (panel B). A higher magnification view of three somatically transformed *E. kuehniella* larvae showing DsRed expression in tissues from the anterior to the posterior (panel C). The posterior regions of a somatically transformed and a nontransformed second instar larva of *T. ni* viewed with Texas red filter (panel D). High levels of DsRed expression can be observed in the posterior fat body and cardiac cells of the somatically transformed larva. The nontransformed larva shows the background level of autofluorescence in the tissues and gut of normal larvae. An anterior view of a transformed larva shows the high level of DsRed expression in the cardiac cells along the dorsal aorta and crop (panel E). Abbreviation: NT, nontransformed control.

recombinant genome into viral particles using a helper virus, recombinant particles were found to transform insect cells and to support P9-regulated expression of the chimeric protein (Giraud *et al.*, 1992). Similarly, the densovirus of *Aedes aegypti* was developed as a gene transfer vector that was used to transduce genes into larvae of *Ae. aegypti* and *Anopheles gambiae* by typical routes of infection (Ward *et al.*, 2001). The vector was deleted for the capsid gene and a chimeric gene including the NS1 and GFP was constructed (Afanasiev *et al.*, 1999). Infection from transducing recombinant viral particles resulted in expression of the chimeric NS1-GFP protein in anal papillae and fat body (Ward *et al.*, 2001). The common drawbacks of these systems are that the vector requires packaging as a transducing viral particle and that the expression patterns are limited to the cell types that viral particles infect.

Other misexpression systems, such as pseudotyped pantropic retroviruses (Jourdan *et al.*, 1990; Matsubara *et al.*, 1996; Franco *et al.*, 1998; Teysset *et al.*, 1998), recombinant Sindbis virus (Kengaku *et al.*, 1998; Lewis *et al.*, 1999) or baculoviruses (Oppenheimer *et al.*, 1999), have also proved to be useful for the analysis of gene function in non-model organisms. However, these systems have limitations in their applications because Sindbis- and retroviral-based vectors are classified as Biosafety Level-2 agents (Jourdan *et al.*, 1990; Matsubara *et al.*, 1996; Franco *et al.*, 1998; Kengaku *et al.*, 1998; Teysset *et al.*, 1998; Lewis *et al.*, 1999) and injection of baculoviruses into many species of Lepidoptera leads to productive infection causing severe cytopathological effects (Lewis *et al.*, 1999; Oppenheimer *et al.*, 1999). Although they are based on the use of viral DNA sequences, *Jc*DNV-derived somatic transformation vectors are radically different in principle to virus-mediated gene transfer systems utilizing transducing viral particles. Unlike virus-mediated transductions, the *Jc*DNV-based system relies strictly on the use of plasmid DNA. Consequently, somatic transformation experiments are performed by direct injection of purified pBRJ-derived plasmid DNA into syncytial embryos, or by transfection into cultured cells and do not require expertise in virology.

Previously, the isolation and characterization of integration sites resulting from *Jc*DNV vector transformation of SF9 chromosomes showed that recombination occurred preferentially within the P9ITR (Bossin *et al.*, 2003) and resulted in integration of most of the plasmidic sequence (Bossin *et al.*, 2003). Removal of viral sequences that eliminated production of the NS3 or all NS proteins did not prevent integration but influenced the number of integrated copies and their distribution in the host genome (Royer *et al.*, 2001; Bossin *et al.*, 2003). In the presence of all NS proteins, *Jc*DNV integration was observable at a single identifiable site within the chromosomes of transformed SF9 cells (Bossin *et al.*, 2003). Disruption of NS gene expression resulted in multiple integration sites and in head-to-tail concatemerization of the plasmid. The present work confirms that the lack of NS expression does not block somatic transformation in either *D. melanogaster* or *P. interpunctella* embryos. Additionally, the data show that removal of the P93ITR does not prevent transformation (Tables 1 and 2). Insertion of a second reporter cassette either within (hr5IE1DsRed) or outside (PUb-nls-EGFP) (Handler & Harrell, 2001) the densoviral sequences did not

affect the rate of somatic transformation either. As found with the pJDR-3xP3GΔNSΔH construct, integration into the host cell chromosomes does not require the presence of NS proteins or the P93ITR as these genetic elements have been eliminated from this construct.

The microinjection protocol was designed to introduce the *Jc*DNV-derived plasmid DNA solution into very early syncytial embryos where few dividing nuclei were present. Expression patterns in late larvae, pupae and adults observed following integration of the various expression cassettes incorporated in the vectors tested demonstrated that somatic transformation occurred rapidly following microinjection and that the frequency of integration was high. Mosaic patterns of GFP expression in the eyes of adult somatically transformed *D. melanogaster* (Fig. 2E,F) indicated that most if not all of the syncytial nuclei carried an integrated copy of the *Jc*DNV vector. The expression patterns in all four species tested extended from the anterior to the posterior of transformed insects and corresponded to expected tissue and temporal patterns for each respective promoter. For example, GFP was observed only in the eyes of adult *D. melanogaster* and *P. interpunctella* that were transformed with *Jc*DNV vectors containing the 3xP3EGFP cassette (Figs 2E,F, 3J). This was in contrast to somatic transformants from *piggyBac* vectors, which displayed spotty GFP expression in areas where 3xP3-driven expression was not expected (Fig. 3K). The specific, persistent expression patterns observed throughout development and into the adult stage reflect the stable integration of the *Jc*DNV vectors. Because integration occurred in all the species tested and the fidelity of transgene expression was not affected by the presence or absence of flanking viral sequences, the *Jc*DNV somatic transformation system appears to be very reliable and should be applicable to a broad range of insects.

*Jc*DNV vectors were used to establish phenotypes for four different promoters while concurrently assessing the activity of multiple transgene expression cassettes incorporated into a single construct. The P9 densovirus promoter, present in all *Jc*DNV vectors used in these experiments, was highly active in all three of the lepidopteran and the one dipteran species tested. The majority of the P9-driven fluorescence was found in the fat body, Malpighian tubules and midgut of all of the insects (data not shown) and corresponded with a previous report (Royer *et al.*, 2001). In addition to expression in these tissues, the cardiac cells of the lepidopteran larvae also supported a very high level of P9-driven expression, which was not observed in the *D. melanogaster* larvae. Similar expression patterns were observed in both *D. melanogaster* and *P. interpunctella* using the hr5IE1 baculovirus enhancer/promoter (Jarvis *et al.*, 1996) to drive DsRed expression. Utilization of either of these two viral promoters in expression cassettes provides reliable evidence of transformation in the insects tested here.

The polyubiquitin promoter (PUb) – driven expression in *D. melanogaster* larvae, pupae and adults from JcDNV was canonical to that observed for *piggyBac* germline transformants (Handler & Harrell, 2001). Although PUb-driven GFP expression was observed in late *P. interpunctella* embryos, GFP was not detected in the postembryonic stages of *P. interpunctella*, even though a high level of P9 expression was observed which confirmed successful somatic transformation. These results are probably because of transient expression from plasmid taken up by vitellophages, as DsRed and GFP fluorescent material was observed in the guts of late embryos and early larvae which then disappeared as feeding began after larval hatch (data not shown). These observations are consistent with transient GFP expression in embryos of the potato tuber moth, *Phthorimaea operculella* (Mohammed & Coates, 2004) following microinjection of a *piggyBac*-PUb-nls-EGFP vector (Handler & Harrell, 2001). The 3xP3 artificial *Pax*6 binding site (Berghammer *et al.*, 1999) was again very active in driving expression in *D. melanogaster* larvae, pupae and adults but was only minimally active in the Lepidoptera tested. Of the somatically transformed *P. interpunctella* or *T. ni* specimens observed, only 0.02% of those positive for P9-DsRed expression also showed 3xP3-EGFP-driven expression. Again, while transient expression from the 3xP3 cassette was observed in the embryos, limited GFP expression was detected in postembryonic stages. These findings indicate that the PUb promoter and 3xP3 artificial binding site do not support highly active transcription in the pyralid and noctuid moths tested. The use of the PUb promoter and 3xP3 artificial binding site in expression cassettes as markers of germline transformation in these and other species of Lepidoptera should therefore be considered with caution.

In comparison with *Jc*DNV vectors, the *piggyBac* vectors containing identical expression cassettes resulted in a frequency of somatic transformation that was at least one order of magnitude lower. The tissue-specific expression from the transgenes within the *piggyBac* vectors was altered as well (compare Fig. 3K and L). These findings imply that somatic integrations from *piggyBac* do not occur as early as those for *Jc*DNV vectors, even though the microinjections of the two vectors were performed at the same stage of embryogenesis. When compared with *Jc*DNV vectors, the *piggyBac* vectors are clearly not reliable somatic transformation vectors.

This work demonstrates that *Jc*DNV plasmids provide stable, efficient somatic transformation vectors for utilization in assessing chromosomally-based gene expression in insects. Using the inherent P9 densovirus promoter to regulate reporter cassette expression, previous work demonstrated that these vectors were integrated into the chromosomes of the host cells (Royer *et al.*, 2001; Bossin *et al.*, 2003), and that when introduced into embryos the integrations persisted through the adult stage (Royer *et al.*,

2001). We have extended these findings and show here that somatic transformation occurs with high efficiency in the four species examined and that expression patterns for additional transgene reporter cassettes retain specific tissue and developmental patterns, regardless of whether they are inserted within or external to the densoviral sequences. The mechanisms of recombination that allow integration of *Jc*DNV vectors into host cell chromosomes and the regulation of integration site recognition remain to be elucidated. Based on the features described here, the *Jc*DNV vectors represent an extremely important and useful tool for the rapid spatiotemporal assessment of gene expression in insects.

Experimental procedures

Insect strains

The *D. melanogaster w*[m] strain contained a *white* mutation with an M cytotype. The strain was obtained from the Bloomington Stock Center (Indiana University, Bloomington, IN) and reared on a standard cornmeal–yeast–molasses diet at 23 °C. The Indian meal moth, *P. interpunctella* (Hübner) (Lepidoptera: Pyralidae) *w* strain contains an autosomal recessive white eye mutation and was reared according to Silhacek & Miller (1972) in a 16 h light: 8 h dark cycle at 30 °C and 70% relative humidity. The Mediterranean meal moth, *E. kuehniella* (Keller) (Lepidoptera: Pyralidae), is a wild-type strain and was reared on the same diet as *P. interpunctella* (Silhacek & Miller, 1972) in a 16 h light: 8 h dark cycle at 26 °C and 70% relative humidity. The cabbage looper, *T. ni* (Hübner) (Lepidoptera: Noctuidae) is a wild-type strain and was reared on a pinto bean-based artificial diet according to the procedures of Guy *et al.* (1985). *T. ni* pupae were sexed and placed in screen cages within an environmental chamber at 26 °C and 70% RH for eclosion and mating. Adult *T. ni* had access to cotton balls saturated with either distilled water or a honey–sugar solution.

Plasmids

Initially, pJDsRedH was created by replacing the 734 bp *Age*I-*Not*I fragment of pJGFPH (Bossin *et al.*, 2003) that contains the GFP coding sequence with the 693 bp *Age*I-*Not*I fragment that contains the DsRed coding sequence from the pDsRed-N1 plasmid (Clontech Laboratories, Inc., Mountain View, CA, USA). In this construct, the DsRed coding sequence is in frame with the VP coding sequence of *Jc*DNV and under the regulation of the P9 viral promoter. To insert the 3xP3EGFP expression cassette into pJDsRedH, a 1432 bp PCR amplicon was produced using the primers *Afl*II-Pax6GFPF 5′-CTTACCTTAAGAACCGTATAAGTTCGAGAT-3′ and *Afl*II-Pax6GFPR 5′-CGGTTCTTAAGAGACGAGAGTAAGGGGTCCGT-3′ with the pB[3xP3EGFP]af (Horn & Wimmer, 2000) template and cloned into pCR2.1 (Invitrogen, Carlsbad, CA, USA). The 1422 bp *Afl*II fragment containing the 3xP3EGFP amplicon from the pCR2.1–3xP3EGFP plasmid was excised and the ends were filled using Klenow (Promega, Madison, WI, USA). The fragment was inserted into the Klenow-filled *Afl*II site (bp 2995) of pJDsRedH to produce pJDR-3xP3G. A pJDsRed plasmid containing a multiple cloning site was constructed by eliminating the *Not*I fragment from pJDR-3xP3G which left unique *Fse*I and *Asc*I restriction sites in pJDsRedafH. The 335 bp *Asc*I-MCS from pSLfa1180fa (Horn & Wimmer, 2000) was then inserted into the *Asc*I site of pJDsRedafH to produce pJDsRedMCSH (pJDR). To provide a vector with the GFP reporter, the 735 bp *Age*I-*Not*I fragment from pEGFP-N1 plasmid (Clontech Laboratories, Inc.) was inserted into the *Age*I-*Not*I site of pJDsRedafH to produce pJGFPafH. The MCS was inserted into pJGFPafH by ligating the 329 bp *Asc*I-MCS-*Not*I fragment from pJDR into the *Asc*I-*Fse*I site to produce pJGFPMCSH (pJDG).

To construct plasmid pJDR-3xP3GΔNS3H, pJDR-3xP3G was digested with *Nsi*I and religated, thus deleting a 112 bp fragment that abolished translation of the NS3 protein. To construct plasmid pJDR-3xP3GΔNSH, pJDR-3xP3G was digested with *Swa*I and religated. This removed a 1389 bp fragment that eliminated translation for all three NS proteins. The pJDR-3xP3GΔNSΔH construct was derived from pJDR-3xP3G by digestion with *Nde*I and religated to remove a 2945 bp fragment including a portion of the NS coding sequence, the P93ITR and a portion of the pBR322 backbone.

The PUb-nls-EGFP expression cassette (Handler & Harrell, 2001) was inserted into pJDsRedH by first inserting a 42 bp linker containing *Fse*I and *Asc*I restriction sites into the *Pvu*II site at bp 7481 located within the pBR322 backbone to produce pJDsRedH-fa. A 4220 bp PUb-nls-EGFP PCR amplicon was generated from the pB[PUb-nls-EGFP] template using the primers *Fse*I-PUbnlsegfp 5′-CACGAGGGCCGGCCGAGTCTCTGCACTGAACATTGTCAG-3′ and *Asc*I-PUbnlsegfp 5′-CCGGTATGGCGCGCCTGTTTGATCGCACGGTTCCCACAA-3′. Following digestion of the pJDsRedH-fa plasmid and the *Fse*I-PUb-nls-EGFP-*Asc*I amplicon with *Fse*I and *Asc*I, the vector and fragment were ligated together to produce pJDsRedH-PUb-nls-EGFP (pJDR-PUbG).

The *Jc*DNV sequence for the P9ITR-DsRed promoter/coding sequence was inserted into the pB[3xP3EGFP]af (Berghammer *et al.*, 1999) vector. A PCR fragment for the P9ITR-DsRed expression cassette was amplified from the pJDR template using the primers FseIHP9 (5′-TGGATGCGGCCGGCCTAGGCTTGGTTATGCCGGTACT-3′) and BglIIMCS (5′-AATTCGAATGGCCATGGGACGTCG-3′). The pB[3xP3EGFP]af plasmid was digested with *Fse*I and *Bgl*II to remove a 761 bp fragment and then the 5′ ITR-P9DsRed fragment was digested with *Fse*I and *Bgl*II and inserted to produce pB[P9DsRed-ITR-3xP3EGFP] (pB-P9R-3xP3G). The phsp-pBacwc helper was constructed by digesting phsp-pBac (Handler *et al.*, 1998) with *BsiW*I and *Bcl*I, creating blunt ends with T4 DNA polymerase and religating the plasmid.

The hr5IE1DsRed expression cassette was constructed by amplifying a PCR product containing the hr5IE1 enhancer/promoter using pACP(+)IE1EGFP (Jarvis *et al.*, 1996) as the template with 5′*Hin*dIIIhr5 (5′-TTTTAAGCTTCTCCATGATGGGCGCGT-3′) and 3′IE1BglII (5′TTGAGCTCGAGATCTAGATCC-3′) as primers. The hr5IE1 PCR product was cloned into pGEM-T Easy (Promega). The 1114 bp *Hin*dIII-*Bgl*II fragment isolated from pGEM-hr5IE1 was inserted into pDsRed-N1 (Clontech Laboratories, Inc.) following digestion with *Hin*dIII-*Bam*HI to produce phr5IE1DsRed. The 2400 bp *Bgl*II-*Ngo*MIV fragment from phr5IE1DsRed was inserted into the pB[3xP3EGFP]af plasmid following digestion with *Bgl*II-*Ngo*MIV and removal of the 764 bp fragment to produce pB[hr5IE1DsRed-3xP3EGFP] (pB-hr5IE1R-3xP3G). Additionally, the 2438 bp *Xho*I-*Asc*I hr5IE1DsRed expression cassette fragment from pGEM-hr5IE1DsRed was inserted into the *Xho*I-*Asc*I sites of pJGFPMCSH to produce pJGFP[hr5IE1DsRed] (pJDG-hr5IE1R).

Embryo injections, somatic transformation and marker detection

In order to achieve optimal exposure of the embryonic nuclei to the plasmid DNA solutions and favour a high probability of achieving

chromosomal integration in syncytial nuclei, the embryos of all species were collected and microinjected just after egg deposition. For *D. melanogaster*, *P. interpunctella* and *E. kuehniella*, the eggs were collected within 7 min of oviposition. Without being dechorionated, the eggs were picked up with a wet paintbrush and aligned with the same orientation on cellophane strips (Bio-Rad, Hercules, CA, USA) for 7 min. The microinjections were conducted utilizing an injection system similar to that described by Handler (2000). The microinjections for a strip of eggs were completed within another 7 min, keeping the age of an embryo between 14 and 21 min at the time of exposure to transforming DNA. Because *T. ni* embryos take longer before cellularization takes place, the eggs were collected, mounted on double stick tape and microinjected within 3 h of oviposition. Following the microinjection of a strip of eggs, the eggs were sealed with a small drop of Krazy® glue (Elmer's Products, Inc., Columbus, OH). For *D. melanogaster*, the cellophane strip was hydrated and then placed on *Drosophila* diet in a 25 mm Petri dish at 21 °C and 100% humidity for development. The embryos of the other species were placed in modular incubators (Billups-Rothenberg, Inc., Del Mar, CA) at 22 °C and 95% humidity until hatching and then transferred to an appropriate diet.

Transforming DNA solutions were prepared in injection buffer (5 mM KCl; 0.1 mM sodium phosphate pH 6.8). The *Jc*DNV-derived plasmids were microinjected at 1 μg plasmid DNA/μl unless otherwise indicated. The *piggyBac* vectors and phsp-pBacwc helper were microinjected at 600 : 400 ng DNA/μl unless otherwise indicated. This microinjection protocol resulted in low hatch rates (5–30%) for *D. melanogaster* as compared with other reports (Rubin & Spradling, 1982), but assured exposure of syncytial nuclei to transforming DNA during early development when only a limited number of nuclei were present. This was especially important when conducting the somatic transformation with *Jc*DNV vectors.

The embryos, larvae, pupae and adults were scored for somatic transformation by determination of fluorescent protein expression. The EGFP and DsRed fluorescent proteins were detected using a Leica MZ FLIII fluorescent stereozoom microscope (Leica Microsystems, Heerbrugg, Switzerland) using a GFP/DsRed dual filter set (Excitation 470/30 nm and 565/20 nm; Barrier 510 and 580 nm; Emitter 535/45 nm and 610/60 nm), a Texas Red filter set (Excitation 560/40 nm; Barrier 610 nm; Emitter 630/60 nm) or a GFP Plus filter set (Excitation 480/40 nm; Barrier 510 nm; Emitter 525/50 nm). Digital photographs of the insects were made using a Leica DC 500 CCD camera operated with Leica IM50 software. In order to detect fluorescence, the insects were removed from diet and viewed individually using the appropriate filter set.

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